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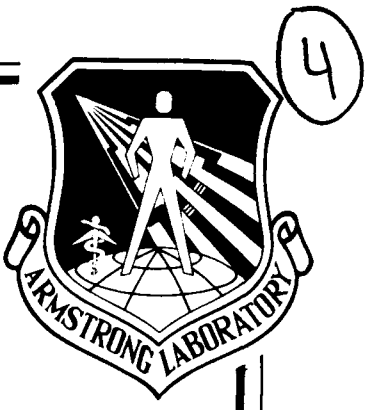

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ARMSTRONG LABORATORY

EVALUATION OF N-METHYL-N'-NITROGUANIDINE IN SHORT-TERM GENETIC TOXICITY TESTS

C. S. Godin

MANTECH ENVIRONMENTAL TECHNOLOGY, INC.
P.O. Box 31009
DAYTON, OH 45431-0009

A. Thilagar

SITEK RESEARCH LABORATORIES
12111 PARKLAWN DRIVE
ROCKVILLE, MD 20852

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

JAMES N. McDOUGAL, Lt Col, USAF, BSC
Deputy Director, Toxicology Division
Armstrong Laboratory

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N-Methyl-*N'*-nitroguanidine (MNG) is being considered as a component of Air Force explosive formulations. This chemical was evaluated in several bioassays to assess its potential genotoxic activity. The assays conducted were the Ames *Salmonella*/microsomal assay, the Chinese hamster ovary/hypoxanthine-guanine phosphoribosyltransferase forward mutation assay, and the mouse *in vivo* bone marrow micronucleus assay. Both *in vitro* assays were conducted in the presence and absence of S9 metabolic activation enzymes. The results of the three genetic tests indicated that MNG does not interact with genetic material. Also, no toxicity was observed for very high dose levels. These assays would therefore predict essentially no genotoxic risk from exposure to MNG.

14. SUBJECT TERMS

Ames Assay, Forward Mutation Assay, Genotoxicity, *N*-Methyl-*N'*-Nitroguanidine, Mouse Micronucleus Assay

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PREFACE

The research reported in this document was conducted by SITEK Research Laboratories under a subcontract to ManTech Environmental Technology, Inc. (ManTech Environmental) in support of the Toxic Hazards Research Unit (THRU). The THRU is the contractor-operated effort of the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory, located at Wright-Patterson Air Force Base, OH. During the initiation and conduct of these studies, Lt Col James N. McDougal served as the Contract Technical Monitor. The experimental work reported here was begun on 11 June 1991 and was completed 8 October 1991 under U.S. Air Force Contract No. F33615-90-C-0532 (Study No. F01).

The genotoxicity assays were conducted at SITEK Research Laboratories in Rockville, MD. These studies were performed in compliance with the Good Laboratory Practice (GLP) regulations for nonclinical laboratory studies and the GLP standards for health effects as described in 21 CFR Part 58 and 40 CFR parts 792 and 160, respectively, except for the stability of the test article and control substances under the experimental conditions which was not determined by SITEK Research Laboratories. The results of their work were reported to ManTech Environmental in separate reports on each assay. The final reports received from SITEK Research Laboratories, copies of the raw data, Quality Assurance Statements, and Good Laboratory Practice Compliance and Certification Statements for each of the assays have been archived in the Quality Assurance Archive of the THRU.

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ABBREVIATIONS

CE	Cloning efficiency
CHO	Chinese hamster ovary
DMBA	7,12-Dimethylbenzene[*a*]anthracene
DMSO	Dimethylsulfoxide
EMS	Ethylmethanesulfonate
FBS	Fetal bovine serum
GLP	Good Laboratory Practice
HBSS	Hank's balanced saline solution
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
HIFBS	Heat inactivated fetal bovine serum
Hx-free	Histidine-free
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MNG	*N*-methyl-*N'*-nitroguanidine
MPCE	Micronucleated PCE
NADP	Nicotinamide-adenine dinucleotide phosphate
NCE	Normochromatic erythrocytes
PCE	Polychromatophilic erythrocytes
RCE	Relative cloning efficience
TEM	Triethylenemelamine
TG	6-Thioguanine
THRU	Toxic Hazards Research Laboratory

SECTION 1

INTRODUCTION

N-Methyl-*N*'nitroguanidine (MNG) is being considered as a component of Air Force explosive formulations. However, little information has been found in the literature concerning either the health hazards or the genotoxic potential of this chemical.

Hsie et al. (1987) found that MNG was not mutagenic in the forward mutation assay and did not produce chromosomal aberrations at concentrations as high as 2000 µM (~240 µg/mL); however, a marginally detectable increase in the frequency of sister chromatid exchanges was observed at concentrations of 1250 µM (~150 µg/mL). Nagao et al. (1975) concluded that MNG was not mutagenic in the *Salmonella* reverse mutation assay (Ames assay) but Ishidate (1988) reported that MNG was mutagenic in the Ames assay and induced chromosomal aberrations at concentrations ranging from 3 to 6 mg/mL.

A battery of tests designed to determine the genotoxic potential of MNG were conducted because (1) the data on MNG mutagenicity had been obtained by different laboratories under different test conditions and thus the results were inconsistent and unreliable; and (2) no *in vivo* mutagenicity assays had been conducted, to our knowledge. This battery of tests consisted of the *Salmonella*/microsomal reverse mutation assay, preincubation method (Ames assay), the Chinese hamster ovary (CHO)/hypoxanthine-guanine phosphoribosyltransferase (HGPRT) forward mutation assay, and the mouse bone marrow micronucleus assay. These assays were chosen because of their high sensitivity and reliability.

The Ames assay was designed to evaluate MNG and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes (S9). The Ames assay detects both base pair substitution point mutations and/or frameshift mutations, and evidence has suggested that a high percentage of chemicals that elicit a mutagenic response in the Ames assay are potential animal and human mutagens and carcinogens (McCann et al., 1975; McCann and Ames, 1976; Sugimura et al., 1976; Tennant et al., 1987). Because the *Salmonella* assay has been shown to indicate mechanisms of chemical interaction with DNA, and has produced few false positives for noncarcinogens, it has been used as the cornerstone of any battery of genotoxicity testing.

The CHO/HGPRT forward mutation assay has been used to determine the mutagenicity of chemicals both in the presence and absence of S9, and has been found to be sensitive to a large

variety of chemicals believed to be mutagens (Hsie et al., 1979, 1981; O'Neill et al., 1977). The gene for HGPRT is located on the X-chromosome. Chinese hamster ovary cells from female hamsters have two X-chromosomes, only one of which is actively transcribed and used in cellular processes. Therefore, two phenotypes exist (1) HGPRT +, in which the enzyme is produced so that hypoxanthine and guanine can be utilized for DNA synthesis; and (2) HGPRT-, in which the enzyme is either not produced or is defective, and the cell cannot use purine or purine analogs for DNA synthesis. 6-Thioguanine (TG), an analog of hypoxanthine and guanine, is toxic to HGPRT + cells when metabolized by HGPRT. HGPRT-mutant phenotypes cannot metabolize TG and survive when grown in culture medium containing TG.

This assay has been routinely included in batteries of tests to define genotoxicity of many industrial and environmental chemicals. The recent U.S. Environmental Protection Agency Gene-Tox Phase III review (Li et al., 1988) revealed that the assay could detect mutagenicity of agents from 25 chemical classes, and, of the 43 known carcinogens tested, 40 were found positive. The review by Li et al. (1988) supports the use of this assay from a standpoint of sensitivity.

The mouse bone marrow micronucleus assay is a rapid, *in vivo* cytogenetic assay based on the observation that cells with broken chromosomes or impairment of the spindle apparatus often have disturbances in the distribution of chromatin during cell division. Micronuclei are formed from chromosomes or chromosome fragments left behind during anaphase and can be scored during interphase (Schmid, 1976). In this assay, polychromatophilic erythrocytes (PCEs) in the bone marrow are scored for the presence of micronuclei. During maturation from erythroblast to erythrocyte the nucleus is extruded, whereas micronuclei, if present, remain in the cytoplasm. Detection of the micronuclei in nonnucleated cells is thus facilitated and provide a useful index of clastogenicity or anaphase-lag in erythroblasts (Schmid, 1976; MacGregor et al., 1980, 1983; Heddle et al., 1983).

SECTION 2

MATERIALS AND METHODS

CHEMICALS

N-Methyl-*N*'nitroguanidine (CAS# 4245-76-5, M.W. 118.12) was obtained from the Air Force Armament Lab (Eglin Air Force Base, FL). The purity of this material was >99% (Brashear et al., In Preparation). This chemical is a white powder that is considered a flammable solid when wet and an explosive when dry. Therefore, MNG was maintained in a moist state.

The positive control agents in the Ames assay were aminoanthracene and sodium azide (Sigma Chemical Co., St. Louis, MO) and 9-aminoacridine and 2-nitrofluorene (Aldrich Chemical Co., Milwaukee, WI). Ethylmethanesulfonate [EMS], (Eastman Kodak, Rochester, NY) and 7,12-dimethyl-benz[a]anthracene [DMBA], (Sigma) were the positive controls for the CHO/HGPRT forward mutation assay. The positive control for the mouse bone marrow micronucleus assay was triethylenemelamine (Radian, Inc., Austin, TX).

MNG was completely solubilized in dimethylsulfoxide (DMSO; Fisher Scientific) to prepare the dosing solutions for both *in vitro* assays. Corn oil was used as the vehicle to administer MNG to mice.

SALMONELLA TYPHIMURIUM PREINCUBATION (AMES) ASSAY

The experimental materials, methods, and procedures are based on those described by Ames et al. (1975) and Maron and Ames (1983).

Bacterial Strains: The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 were obtained from Dr. Bruce N. Ames, University of California, Berkeley. Overnight cultures of each strain were prepared by transferring a sample of each frozen strain into a shaker flask containing approximately 50 mL of sterile Oxoid Nutrient Broth No. 2. The flask was placed in a shaker-incubator for 8 to 12 h at a temperature of 37 ± 1 °C. The cultures were removed from the shaker incubation when they reached a density of 5.0×10^8 to 1.0×10^9 cells/mL. On the day they were required for use in the assay, all tester strain cultures were tested for histidine requirement and the *rfa* wall mutation. Strains TA98 and TA100 also were tested for the presence of the pKM101 plasmid. The spontaneous reversion frequency of each strain was examined by plating aliquots of each strain along with the vehicle.

S9 Metabolic Activation Mixture: S9 liver fraction was prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 at a dose of 500 mg/kg. The procedure employed was based on that described by Ames et al. (1975). The components of the S9 activation mixture were

8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM nicotinamide-adenine dinucleotide phosphate (NADP), 100 mM sodium phosphate (pH 7.4), and 4% S9 liver fraction.

Dose Rangefinding Test: In order to determine the appropriate test article concentrations for the mutation assay, a dose rangefinding test was performed using tester strain TA100 both in the presence and absence of S9. One plate for each of seven doses of MNG ranging from 5 to 5000 µg per plate was prepared, as well as a plate for the vehicle control. Cytotoxicity was indicated by a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn and was scored relative to the vehicle control plate. Routinely, the maximum dose selected for subsequent testing in the mutagenicity assay should be cytotoxic, if possible.

Mutation Assay: Doses were selected for the mutation assay on the basis of the results of the rangefinding test. The assay was conducted using three plates per dose in both the presence and absence of S9. Five doses of MNG were tested ranging from 1000 to 5000 µg per plate. Three plates were also prepared DMSO and the positive control. The same doses were used in a second assay that was performed to confirm the results of the first assay.

The assay was initiated by adding either 500 µL of water or 500 µL of S9 mix to a series of labeled culture tubes. Immediately thereafter, 100 µL of the appropriate tester strain was added, followed by 100 µL of the appropriate dose of MNG, solvent, or positive control. Each tube was vortexed and incubated at 37 ± 1 °C for 20 min with gentle shaking. After the incubation period, 2 mL of top agar (containing histidine and biotine) was added to all the culture tubes. The contents were mixed by vortexing, and the contents were evenly distributed over a Vogel-Bonner bottom-agar plate. Each plate was placed on a level surface until the top agar solidified. The plates were inverted and incubated at 37 ± 1 °C for 48 to 72 h. After the incubation, the plates were removed from the incubator, and the colonies were counted using an automatic colony counter (Artek Counter, model 880). Automatic colony counting was performed by making three counts per plate and the mean count per plate was recorded.

A response was considered positive if at least one dose produced a mean reversion frequency in a given strain that was at least two times greater than the mean reversion frequency of the corresponding vehicle control plates, and the response was dose-dependent.

CHO/HGPRT FORWARD MUTATION ASSAY

Indicator Cells: The CHO cell line used in this study was obtained from Dr. Patrick O'Neill, University of Vermont. Master stocks were maintained frozen in liquid nitrogen. The cells were propagated in Ham's F-12 culture medium to obtain sufficient cells for use in the assay.

To reduce the spontaneous frequency of HGPRT-mutants, the cell cultures were cleansed prior to freezing. The cleansing medium consisted of Ham's F-12 medium supplemented with 3 µM thymidine, 3 µM hypoxanthine, 100 µM glycine, and 10 µM aminopterin. The cells remained in this medium for 24 h. The cells were then refed with medium that did not contain aminopterin.

After cleansing, the cells were cryopreserved in Ham's F-12 medium containing 8% DMSO. These stock cultures were tested for karyotype stability and mycoplasma contamination prior to use in the mutagenicity assay. The CHO stock cultures were maintained in complete histidine-free (Hx-free) medium supplemented with 10% heat inactivated fetal bovine serum (HIFBS), 2 mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL of streptomycin, and grown in T-75cm^2 tissue culture flasks at 37 ± 1 °C. Cells were subcultured just before reaching confluency.

Preparation of Test Cultures: The CHO stock cultures were harvested when a confluency of approximately 50 to 90% had been reached and they were then used to prepare the test cultures. The cells from a number of stock culture flasks were removed and pooled to obtain a single suspension. An aliquot of the suspension was counted and, based on the cell counts, an appropriate amount of complete Hx-free culture medium was added to yield a suspension containing 1 x 10^5 cells/mL. This dilution was used to seed the test flasks. An appropriate number of T-25 cm^2 plastic culture flasks were seeded with 5 mL of the cell suspension to obtain test cultures containing 5 x 10^5 cells/flask. The flasks were incubated at 37 ± 1 °C in a humidified incubator in an atmosphere of approximately 5% CO_2 and 95% air for 18 to 24 h.

S9 Preparation: Liver S9 was prepared as described above for the Ames assay. Immediately prior to treatment, the S9 fraction was mixed with cofactors to obtain the S9 cofactor mix, which was kept on ice until used. The S9 cofactor mix consisted of 50 mM sodium phosphate (pH 7.5), 4 mM NADP, 5 mM glucose-6-phosphate, 30 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 100 µL/mL S9 fraction. Prior to its use, the S9 cofactor mixture was diluted 1:5 (v:v) with Hx-free nutrient medium supplemented with 2 mM L-glutamine (serum-free culture medium), and then added to preplated CHO cells.

Dose Rangefinding Assay: A maximum applied concentration of 5000 µg/mL was chosen for preliminary rangefinding cytotoxicity testing. Duplicate cultures of 10 concentrations spanning a 4-log concentration range (0.1 to 5000 µg/mL) were tested both in the presence and absence of S9. Duplicate cultures were also prepared for negative (media) and solvent controls. The cells were exposed to MNG for 5 h, washed with Hank's balanced saline solution (HBSS), and cultured for an additional 18 to 24 h prior to determination of cytotoxicity.

To determine cytotoxicity, the cells from each flask were removed, and the suspensions from cultures exposed to the same concentration of MNG were pooled. The cells were seeded in triplicate

dishes at a density of 200 cells per 60 mm dish. Hx-free medium supplemented with 10% HIFBS, 2 mM L-glutamine, 50 units/mL penicillin and 50 µg/mL streptomycin was used as the culture medium. The cells were permitted to grow for a period of 7 days without disturbance. The colonies were then washed with HBSS, fixed with methanol, stained with Giemsa, and counted. A cluster of more than 50 cells growing within a confined area was considered a colony. The average number of colonies per plate was calculated and the relative cloning efficiency (RCE) was determined by the following formula.

$$RCE = \frac{Average\ No.\ of Colonies\ in\ Test\ Plates}{Average\ No.\ of Colonies\ in\ Solvent\ Plates} \times 100$$

The cytotoxicity was evaluated on the basis of the RCE. A dose causing approximately a 50 to 90% reduction in the RCE has been routinely chosen as the highest dose for use in the mutation assay. In the absence of cytotoxicity, 5 mg/mL has been routinely used as the highest concentration for testing.

Mutation Assay: The test cultures for the mutation assay were seeded approximately 18 to 24 h prior to use and were prepared as described above for the rangefinding assay. Duplicate cultures seeded with 5×10^5 cells/flask were used for each of five concentrations of MNG (ranging from 50 to 5000 µg/mL), as well as for the solvent and negative control. The cells were exposed to MNG for 5 h both in the presence and absence of S9. A confirmatory mutation assay was performed at the same test article concentrations as in the first assay.

After the exposure period, the cells were washed with HBSS, refed with complete Hx-free medium, and permitted to grow for 18 to 24 h. The cells were then used to initiate cultures for the expression of the HGPRT- mutant phenotype (TG-resistant).

For the expression of TG-resistant mutants, the cells from each of the duplicate culture flasks were subcultured in Hx-free Ham's F-12 nutrient medium supplemented with 5% dialyzed HIFBS, 2mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin (cloning medium) at a density of 1×10^6 cells/75 cm^2 flask. The cells were subcultured at 2- to 3-day intervals for a period of 10 days prior to selecting the mutant phenotypes. After this expression period, the cells from each of the duplicate dishes were harvested and seeded in five 100 mm^2 tissue culture plates at a density of 2×10^5 cells/plate in 10 mL of cloning medium containing 10 µM of TG.

To determine the RCE of the cells at the time of mutant selection, 200 cells/60 mm^2 dish were concurrently plated in triplicate in the cloning medium. All clonable test doses and appropriate positive and solvent controls were cultured under mutant selection conditions. The cultures were incubated for 7 days without disturbing the plates to minimize the formation of satellite colonies.

The colonies were then washed with buffered saline, fixed, stained with Giemsa, and counted to determine RCE.

The average number of mutant clones from the duplicate plates in the mutation assay was calculated, and the percent clonable cells (cloning efficiency [CE]) for each treatment condition was determined. The number of TG-resistant mutants for 1×10^6 cells seeded was calculated by totaling the number of mutants from the duplicate plates. Based on the CE, the number of TG-resistant mutants per 1×10^6 surviving cells was calculated for each concentration of MNG.

The results of the assay were evaluated on the basis of the number of TG-resistant mutants per 1×10^6 surviving cells. For statistical analysis, the mutant frequency data were tested for homogeneity of variances among the various groups. The data were subjected to ANOVA and Student's t-tests. If one or more concentrations were found to result in a signficant increase in the mutant frequency then simple regression was performed to determine a dose relationship. A significance level of 0.05 was used in all tests.

The test article was considered to have caused a positive response if (1) the test article showed a positive dose response and at least one test concentration resulted in a statistically significant increase in the number of mutants per 1×10^6 surviving cells, or a two-fold increase in the number of mutants per 1×10^6 surviving cells, or (2) in the absence of a positive dose–response trend, at least two consecutive concentrations of the test article resulted in a significant increase in the number of mutants per 1×10^6 surviving cells.

MOUSE BONE MARROW MICRONUCLEUS ASSAY

Animals: Male and female CD-1 mice (42 days old) were purchased from Charles River Laboratories (Raleigh, NC). The animals received Purina certified rodent chow and fresh tap water ad libitum. A 12-h diurnal light cycle was employed. The animals were randomized and placed into treatment groups each containing five mice of each sex. One such treatment group was designated for each of the test article doses and for the vehicle control and positive control.

Rangefinding Test: Three animals per sex per dose were used. The doses of MNG ranged from 0.1 to 1538 mg/kg. The animals were dosed three times via oral gavage at 24-h intervals. In all treatments the volume administered to the animals was limited to 10 mL/kg. This volume produced no significant toxic effects. Clinical symptoms and body weights were observed and recorded daily for 3 days after treatment.

Micronucleus Assay: The animals were reweighed and placed in weight groups that varied by no more than ± 0.5 g. The animals were then sequentially assigned from the weight groups to randomized cages corresponding to treatment groups consisting of five animals each. The doses of

MNG ranged from 320 to 1600 mg/kg. Each animal was dosed three times via oral gavage at 24-h intervals. A single dose of TEM was administered intraperitoneally at a dose of 1.0 mg/kg as the positive control.

Twenty-four hours after the last dose, five mice per sex were sacrificed from each dose level and from the vehicle control. Five male and five female mice treated with the positive control were sacrificed 24 h after treatment. All animals were sacrificed by CO_2 asphyxiation.

The groin area of each animal was wetted with 70% ethanol, and the femurs were exposed by cutting into the skin and muscle of the thighs. The femurs were separated just above the kneecaps, and the heads of the femurs were removed with bone cutters. The bone marrow from the femurs was flushed into a 15-mL centrifuge tube using a 1-cc syringe fitted with a 25-gauge needle and containing a small volume of fetal bovine serum (FBS). Each 15-mL centrifuge tube contained approximately 1 mL of FBS. After the cells from one group of animals had been collected, the tubes were centrifuged at 1000 rpm for 5 min. The supernatant was removed, leaving approximately 0.1 mL above the cell pellet.

Preparation, Staining, and Scoring of Slides: The cells were resuspended by flicking the tube until a homogeneous suspension was observed. A small drop of the cell suspension was placed just below the frosted end of a precleaned microscope slide and spread along the length of the slide. The slides were fixed with methanol and allowed to air dry at least overnight. The slides were stained in 5% Giemsa diluted with phosphate buffer and were then mounted in Permount using #1 cover glasses. The slides were scored "blind" in order to avoid bias on the part of the scorers. The ratio of PCE to normochromatic erythrocytes in 1000 erythrocytes was determined. In addition, the number of micronucleated PCE (MPCE) per 1000 PCE was determined.

Statistical Analysis: Data from male and female animals were analyzed separately. A two tailed Student's t-test was used for the statistical analysis of data. The test article was considered to have caused a positive response in this assay if (1) the test article resulted in a positive dose–response trend and a statistically significant increase in the number of MPCE over that of the concurrent vehicle control was observed at one or more dose levels, or (2) in the event that there was no positive dose–response trend, at least two consecutive test doses resulted in a statistically significant increase in the number of MPCEs.

SECTION 3

RESULTS

AMES ASSAY

The rangefinding test indicated that the test article was nontoxic at all doses tested, both in the presence and absence of S9 (data not shown).

The results of the first and confirmatory assays are summarized in Tables 1 and 2. Comparison of the solvent control values with those for test article-treated plates in the two assays clearly indicated that the test article produced a negative response for all of the strains both in the presence and absence of S9. Strain TA1538 exhibited a reduction in the background lawn at concentrations of 5000 and 4000 µg/plate, both in the presence and absence of S9, but only in the first Ames assay.

The results for the positive controls and negative controls were within the acceptable range.

CHO/HGPRT FORWARD MUTATION ASSAY

The results of the rangefinding test revealed that MNG was nontoxic at all concentrations tested, both in the presence and absence of S9.

The results of the cytotoxicity testing from the first and confirmatory mutation assays are summarized and presented in Tables 3 and 6. In the first assay, all the test article doses were relatively nontoxic both in the presence and absence of S9. In the confirmatory assay, the highest concentration of 5000 µg/mL had a slightly toxic response. The RCE of this dose was 75% in the absence of S9 and 73% in the presence of S9.

The CE and mutant frequency from the two assays are summarized in Tables 4 and 5 (for incubations conducted in the absence of S9) and Tables 7 and 8 (for incubations conducted in the presence of S9. The results indicated that the test article did not cause a significant increase in the mutant frequency at the HGPRT locus in the presence or absence of S9 activation. None of the test article-treated cultures exhibited a mutant frequency that was significantly elevated over that of either the negative or solvent control. Furthermore, no indication of a positive dose–response was observed. The mutant frequencies of the solvent and untreated controls were within the acceptable range. As anticipated, exposure to the positive controls (EMS and DMBA) caused a significant increase in the mutant frequency.

MOUSE BONE MARROW MICRONUCLEUS ASSAY

A significant loss of body weight was observed 72- to 96-h posttreatment in male mice that were administered MNG at a dose of 1538 mg/kg in the rangefinding test. No clinical symptoms were

observed in any animals. One female mouse had received MNG at a dose of 1000 mg/kg was found dead prior to the second dosing. This was most likely due to the dosing procedure rather than an effect of the MNG because no clinical symptoms or deaths were observed in animals exposed to the higher dose of 1538 mg/kg.

In the micronucleus assay, no clinical symptoms were observed in the animals in any dose group, and no treatment-related deaths occurred. There were no significant changes in body weight in the test article-treated animals relative to the vehicle control animals.

Individual results in the assay for male and female mice are presented in Tables 9 and 10, respectively. There were no statistically significant increases in the number of MPCEs in any test doses compared to the concurrent vehicle control.

TABLE 1. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE FIRST *SALMONELLA TYPHIMURIUM* PREINCUBATION MUTATION ASSAY

Dose (µg/plate)	Average Revertants/Plate[a]									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+ S9	-S9	+ S9	-S9	+ S9	-S9	+ S9	-S9	+ S9
Solvent Control[b]	24	32	79	77	13	13	9	10	10	11
1000	22	30	78	72	12	9	12	8	8	10
2000	32	39	73	74	13	9	8	12	7	12
3000	29	29	76	66	13	9	9	10	10	12
4000	28	33	67	64	11	12	10	11	8	11
5000	31	38	65	63	9	10	8	8	8	12
Positive Control[c]	1441	2136	849	1076	908	236	190	175	1305	454

[a] Average of three plates/dose.
[b] Solvent = DMSO (0.1 mL/plate)
[c] The positive controls for incubations conducted in the absence of S9 were 2-nitrofluorene (5.0 µg/plate) for TA98 and TA1538, sodium azide (1.0 µg/plate) for TA100 and TA1535, and 9-aminoacridine (50 µg/plate) for TA1537. In the presence of S9, the positive control for all strains was 2-aminoanthracene (1.25 µg/plate)

TABLE 2. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE CONFIRMATORY *SALMONELLA TYPHIMURIUM* PREINCUBATION MUTATION ASSAY

Dose (µg/plate)	Average Revertants/Plate[a]									
	TA98		TA100		TA1535		TA1537		TA1538	
	-S9	+ S9	-S9	+ S9	-S9	+ S9	-S9	+ S9	-S9	+ S9
Solvent Control[b]	31	33	78	82	14	18	17	17	11	13
1000	28	37	75	80	12	13	13	14	7	10
2000	35	43	76	92	15	11	15	13	10	12
3000	31	37	77	77	14	14	12	15	12	13
4000	32	34	69	76	14	12	16	17	12	10
5000	28	32	70	75	14	12	15	15	14	15
Positive Control[c]	1646	1772	689	1434	615	178	299	176	1248	881

[a] Average of three plates/dose
[b] Solvent = DMSO (0.1 mL/plate)
[c] The positive controls for incubations conducted in the absence of S9 were 2-nitrofluorene (5.0 µg/plate) for TA98 and TA1538, sodium azide (1.0 µg/plate) for TA100 and TA1535, and 9-aminoacridine (50 µg/plate) for TA1537. In the presence of S9, the positive control for all strains was 2-aminoanthracene (1.25 µg/plate)

TABLE 3. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE FIRST CHO/HGPRT MUTATION ASSAY

CYTOTOXICITY TEST RESULTS							
WITHOUT S9				WITH S9			
Concentration µg/mL	Ave. No of Colonies/ Dose	SD[a]	RCE[b] (%)	Concentration µg/mL	Ave. No of Colonies/ Dose	SD[a]	RCE[b] (%)
Negative Cont.	205	15.5	107	Negative Cont.	188	15.2	98
Solvent Cont.[c]	192	15.5	100	Solvent Cont.[c]	191	17.4	100
50	198	14.7	103	50	199	12.9	104
100	208	16.2	108	100	213	16.1	112
500	184	20.3	96	500	194	4.3	102
1000	194	9.1	101	1000	203	10.8	106
5000	181	5.7	94	5000	175	14.2	92
EMS Solvent Control[c]	192	15.5	100	DMBA Solvent Control[d]	188	14.1	100
EMS (0.5 µL/mL)	66	9.1	34	DMBA (5.0 µg/mL)	78	5.8	41

[a] SD = Standard Deviation

[b] $$RCE = \frac{Average\ No.\ of\ Colonies\ in\ Test\ Plates}{Average\ No.\ of\ Colonies\ in\ Control\ Plates} \times 100$$

[c] Solvent = DMSO (50 µL/flask).

[d] DMBA Solvent = acetone (50 µL/flask)

TABLE 4. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE FIRST CHO/HGPRT MUTATION ASSAY

CLONING EFFICIENCY AND MUTANT FREQUENCY RESULTS (WITHOUT S9)						
Concentration µg/mL		Ave. No. of Colonies/Plate	SD	CE[a] (%)	Total Mutants Counted	Mutants/10^6 Surviving Cells[b]
Negative	A	207	6.1	104	7	7
Cont.	B	217	1.2	109	1	1
Solvent	A	223	9.7	112	8	7
Cont.[c]	B	166	5.0	83	1	1
50	A	231	8.3	116	0	0
50	B	203	9.0	102	12	12
100	A	203	8.2	102	4	4
100	B	212	11.4	106	2	2
500	A	195	11.0	98	8	8
500	B	207	6.4	104	4	4
1000	A	201	14.1	101	2	2
1000	B	201	14.1	101	7	7
5000	A	207	10.6	104	8	8
5000	B	202	11.9	101	5	5
EMS Sol.	A	223	9.7	112	8	7
Cont.[c]	B	166	5.0	83	1	1
EMS	A	134	1.2	67	474	707
(0.5 µL/mL)	B	142	3.6	71	505	711

[a] CE = Cloning Efficiency

[b] $$\text{Mutants}/10^6\,\text{Survivors} = \frac{100}{\text{CE}} \times \text{Total Mutants Counted Per Dose} \times \frac{\text{Mutant Plates Seeded Per Dose}}{\text{Mutant Plates Counted Per Dose}}$$

[c] Solvent = DMSO (50 µL/flask)

TABLE 5. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE FIRST CHO/HGPRT MUTATION ASSAY

CLONING EFFICIENCY AND MUTANT FREQUENCY RESULTS (WITH S9)						
Concentration µg/mL		Ave. No. of Colonies/Plate	SD	CE[a] (%)	Total Mutants Counted	Mutants/10^6 Surviving Cells[b]
Negative Cont.	A	177	8.2	89	2	2
	B	209	15.3	105	6	6
Solvent Cont.[c]	A	219	15.8	110	16	11
	B	206	9.6	103	1	1
50	A	215	18.6	108	3	3
50	B	227	15.5	114	10	9
100	A	198	12.8	99	2	2
100	B	211	18.8	106	5	5
500	A	256	6.4	128	0	0
500	B	231	7.3	116	18	16
1000	A	178	9.6	89	3	3
1000	B	172	9.2	86	12	14
5000	A	223	23.0	112	6	5
5000	B	213	4.8	107	7	7
DMBA Sol. Cont.[d]	A	235	14.7	118	11	9
	B	221	15.9	111	6	5
DMBA (0.5 µL/mL)	A	168	1.7	84	563	670
	B	161	5.7	81	467	580

[a] CE = Cloning Efficiency

[b] $$\text{Mutants}/10^6\,\text{Survivors} = \frac{100}{\text{CE}} \times \text{Total Mutants Counted Per Dose} \times \frac{\text{Mutant Plates Seeded Per Dose}}{\text{Mutant Plates Counted Per Dose}}$$

[c] Solvent = DMSO (50 µL/flask)

[d] DMBA Solvent = acetone (50 µL/flask)

TABLE 6. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE CONFIRMATORY CHO/HGPRT MUTATION ASSAY

CYTOTOXICITY TEST RESULTS							
WITHOUT S9				WITH S9			
Concentration µg/mL	Ave. No of Colonies/ Dose	SD[a]	RCE[b] (%)	Concentration µg/mL	Ave. No of Colonies/ Dose	SD[a]	RCE[b] (%)
Negative Cont.	189	32.0	131	Negative Cont.	155	7.7	99
Solvent Cont.[c]	144	9.0	100	Solvent Cont.[c]	157	19.6	100
50	139	4.7	97	50	136	5.4	87
100	136	8.5	94	100	115	11.2	73
500	128	8.9	89	500	118	4.6	75
1000	125	12.0	87	1000	139	14.7	89
5000	108	4.7	75	5000	115	10.7	73
EMS Solvent Control[c]	144	9.0	100	DMBA Solvent Control[d]	138	6.4	100
EMS (0.5 µL/mL)	39	2.9	27	DMBA (5.0 µg/mL)	46	8.3	33

[a]SD = Standard Deviation

[b] $$RCE = \frac{Average\ No.\ of\ Colonies\ in\ Test\ Plates}{Average\ No.\ of\ Colonies\ in\ Control\ Plates} \times 100$$

[c]Solvent = DMSO (50 µL/flask)

[d]DMBA Solvent = acetone (50 µL/flask).

TABLE 7. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE CONFIRMATORY CHO/HGPRT MUTATION ASSAY

CLONING EFFICIENCY AND MUTANT FREQUENCY RESULTS (WITHOUT S9)						
Concentration µg/mL		Ave. No. of Colonies/Plate	SD	CE[a] (%)	Total Mutants Counted	Mutants/10^6 Surviving Cells[b]
Negative Cont.	A	164	9.2	82	2	2
	B	179	2.6	90	1	1
Solvent Cont.[c]	A	179	12.6	90	3	3
	B	171	10.3	86	1	1
50	A	151	11.2	76	0	0
50	B	170	23.7	85	0	0
100	A	185	10.7	93	6	6
100	B	199	8.6	100	8	8
500	A	198	14.7	99	0	0
500	B	171	9.8	86	4	5
1000	A	180	4.5	90	7	8
1000	B	177	6.6	89	5	6
5000	A	180	5.7	90	1	1
5000	B	179	6.6	90	5	6
EMS Sol. Cont.[c]	A	179	12.6	90	3	3
	B	171	10.3	8.6	1	1
EMS (0.5 µL/mL)	A	147	4.9	74	425	578
	B	150	9.8	75	407	543

[a] CE = Cloning Efficiency

[b] $$\text{Mutants}/10^6\,\text{Survivors} = \frac{100}{\text{CE}} \times \text{Total Mutants Counted Per Dose} \times \frac{\text{Mutant Plates Seeded Per Dose}}{\text{Mutant Plates Counted Per Dose}}$$

[c] Solvent = DMSO (50 µL/flask)

TABLE 8. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE CONFIRMATORY CHO/HGPRT MUTATION ASSAY

CLONING EFFICIENCY AND MUTANT FREQUENCY RESULTS (WITH S9)						
Concentration µg/mL		Ave. No. of Colonies/Plate	SD	CE[a] (%)	Total Mutants Counted	Mutants/10^6 Surviving Cells[b]
Negative Cont.	A	178	10.6	89	3	3
	B	182	8.3	91	1	1
Solvent Cont.[c]	A	218	4.2	109	2	11
	B	173	2.9	87	0	0
50	A	171	17.1	86	0	0
50	B	173	7.3	87	3	3
100	A	173	5.4	87	0	0
100	B	196	14.3	98	1	1
500	A	213	17.3	107	1	1
500	B	206	10.2	103	4	4
1000	A	192	9.4	96	0	0
1000	B	159	8.6	80	8	10
5000	A	181	0.5	91	1	1
5000	B	128	14.7	64	1	2
DMBA Sol. Cont.[d]	A	183	9.5	92	0	0
	B	151	0.8	76	0	0
DMBA (0.5 µL/mL)	A	148	9.7	74	385	520
	B	141	12.0	71	159	376

[a] CE = Cloning Efficiency

[b] $$\text{Mutants}/10^6\,\text{Survivors} = \frac{100}{\text{CE}} \times \text{Total Mutants Counted Per Dose} \times \frac{\text{Mutant Plates Seeded Per Dose}}{\text{Mutant Plates Counted Per Dose}}$$

[c] Solvent = DMSO (50 µL/flask)

[d] DMBA Solvent = acetone (50 µL/flask)

TABLE 9. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE MOUSE MICRONUCLEUS ASSAY (MALE)

RATIO OF PCE/NCE AND INCIDENCE OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES IN BONE MARROW CELLS						
Dose (mg/kg)	Animal Number	Cell Counts PCE	NCE	PCE:NCE	MPCE per 1000 PCE	p Value
Corn oil[a]	6232	469	531	0.88	1	
Corn oil	6233	416	584	0.71	2	
Corn oil	6207	412	588	0.70	1	
Corn oil	6211	542	458	1.18	0	
Corn oil	6217	482	518	0.93	0	
				= = =	= = =	
Mean				0.88	0.8	
				= = =	= = =	
320	6313	456	544	0.84	1	
320	6201	443	557	0.80	1	
320	6238	523	477	1.10	0	
320	6317	490	510	0.96	0	
320	6314	445	555	0.80	1	
				= = =	= = =	
Mean				0.90	0.6[b]	
				= = =	= = =	
800	6227	569	431	1.32	1	
800	6222	513	487	1.05	0	
800	6208	426	574	0.74	1	
800	6228	430	570	0.75	0	
800	6315	373	627	0.59	1	
				= = =	= = =	
Mean				0.89	0.6[b]	
				= = =	= = =	
1600	6226	403	597	0.68	0	
1600	6316	506	494	1.02	0	
1600	6213	439	561	0.78	0	
1600	6218	477	523	0.91	1	
1600	6221	403	597	0.68	2	
				= = =	= = =	
Mean				0.81	0.6[b]	
				= = =	= = =	
TEM[c]	6229	268	732	0.37	94	
TEM	6216	377	623	0.61	42	
TEM	6225	289	711	0.41	126	
TEM	6223	318	682	0.47	68	
TEM	6202	375	625	0.60	70	
				= = =	= = =	
Mean				0.49	80	0.005
				= = =	= = =	

[a] Corn oil used as solvent

[b] t-test not done because mean value of treatment group was lower than control

[c] TEM administered at a dose of 1 mg/kg

TABLE 10. MUTAGENICITY OF *N*-METHYL-*N'*-NITROGUANIDINE IN THE MOUSE MICRONUCLEUS ASSAY (FEMALE)

RATIO OF PCE/NCE AND INCIDENCE OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES IN BONE MARROW CELLS						
Dose (mg/kg)	Animal Number	Cell Counts PCE	Cell Counts NCE	PCE:NCE	MPCE per 1000 PCE	p Value
Corn oil[a]	6264	595	405	1.47	2	
Corn oil	6289	465	535	0.87	0	
Corn oil	6250	437	563	0.78	0	
Corn oil	6260	488	512	0.95	0	
Corn oil	6297	424	576	0.74	2	
	Mean			0.96	0.8	
320	6257	524	476	1.1	0	
320	6254	423	577	0.73	2	
320	6245	461	539	0.86	1	
320	6256	493	507	0.97	2	
320	6296	572	428	1.34	0	
	Mean			1.0	1	0.838
800	6248	486	514	0.95	1	
800	6255	338	662	0.51	1	
800	6299	368	632	0.58	0	
800	6293	473	527	0.90	0	
800	6318	430	570	0.75	0	
	Mean			0.74	0.4[b]	
1600	6249	526	474	1.11	1	
1600	6290	403	597	0.68	2	
1600	6258	508	492	1.03	2	
1600	6291	458	542	0.85	0	
1600	6298	430	570	0.75	0	
	Mean			0.88	1	0.815
TEM[c]	6259	311	689	0.45	74	
TEM	6285	284	716	0.4	64	
TEM	6292	264	736	0.36	46	
TEM	6295	341	659	0.52	31	
TEM	6288	261	739	0.35	62	
	Mean			0.42	55.4	0.001

[a] Corn oil used as solvent

[b] t-test not done because mean value of treatment group was lower than control

[c] TEM administered at a dose of 1 mg/kg

SECTION 4

DISCUSSION AND CONCLUSIONS

N-Methyl-*N'*-nitroguanidine administered at a dose of 1538 mg/kg in the mouse bone marrow micronucleus assay did not result in any clinical signs of toxicity. These data are in agreement with those of Kinkead et al. (1991). In that study MNG was administered at a dose of 1 g/kg and no clinical signs of toxicity were observed. In addition, these authors determined that MNG was not a skin irritant but produced slight eye irritation and was a weak sensitizer in the guinea pig.

The nitrosation of MNG results in the formation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) which is both mutagenic and carcinogenic (Endo et al., 1974). It has been proposed that compounds similar in structure to MNG undergo intragastric reaction with nitrites in the diet to form carcinogenic or mutagenic compounds (Endo et al., 1975). The potential for accidental ingestion of MNG containing MNNG as a contaminant or the conversion of MNG to MNNG following such an exposure exists. However, the sample of MNG used in the present study does not contain detectable quantities of MNNG, and no detectable conversion of MNG to MNNG in the stomach of rodents was noted (Brashear et al., 1991). The latter information is interesting because MNG can be converted to MNNG under acidic conditions in the presence of nitrites (Mirvish, 1975). The failure to detect MNNG in the study by Brashear et al. (1991) may simply reflect a very low concentration of nitrate or nitrite in the diet. The negative findings in the mouse bone marrow micronucleus assay in the present study would tend to suggest that MNNG was not endogenously formed from MNG. However, MNNG may be highly reactive with stomach tissues or may be poorly absorbed systemically. The possibility for the conversion of MNG to MNNG is likely under certain predisposing conditions, and further research should involve studies aimed at determining these conditions and whether systemic absorption of MNNG causes genotoxicity.

The results of the three assays were negative and indicate that MNG does not interact with genetic material. It is therefore unlikely that genetic activity would be revealed by other genetic tests and the results of the three assays would predict essentially no genotoxic risk from exposure to MNG.

SECTION 5

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